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**14. ABSTRACT**
The loss of bulk tissues in limb injuries, including segmental defects, is unfortunately common and results in extensive, complex wounds characterized by loss of several tissue types including, bone, cartilage, and muscle. Treatment of extensive multi-tissue defects poses many reconstructive challenges notably suitable cell sources, supportive 3-dimensional (3-D), spatially relevant matrices, and the engineering of tissues with functional properties appropriate to their biomechanical demands.

• The creation of such tissue replacements can be considered under the term, functional tissue engineering.
• We confirmed our original hypotheses that amniotic stem cells, which are widely available, can differentiate into multiple tissues and that the ability of cells to self-assemble and self-sort in 3-D matrices creates spatially robust tissue aggregates.
• We then investigated the chondrogenic potential of MSCs isolated from the amniotic and chorionic membranes seeded in agarose by assessing responses to growth factor delivery and compressive loading on matrix production.

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## INTRODUCTION

The loss of bulk tissues in limb injuries, including segmental defects, is unfortunately common and results in extensive, complex wounds characterized by loss of several tissue types including, bone, cartilage, and muscle. Treatment of extensive multi-tissue defects poses many reconstructive challenges notably suitable cell sources, supportive 3-dimensional (3-D), spatially relevant matrices, and the engineering of tissues with functional properties appropriate to their biomechanical demands. The creation of such tissue replacements can be considered under the term, *functional tissue engineering*. We confirmed our original hypotheses that amniotic stem cells, which are widely available, can differentiate into multiple tissues and that the ability of cells to self-assemble and self-sort in 3-D matrices creates spatially robust tissue aggregates. We then investigated the chondrogenic potential of MSCs isolated from the amniotic and chorionic membranes seeded in agarose by assessing responses to growth factor delivery and compressive loading on matrix production.

These studies, while demonstrating the feasibility of our approach, raised new interesting questions that we feel need to be studied. Therefore, we requested to use the remaining funds in a no-cost extension to extend observations made in the first two years. This request was approved and a copy of the new statement of work is located in the Appendix.

*Green when task is completed. Light green for task that is on schedule and active. Yellow when it is delayed; include a red line showing when it is to start and when you anticipate it to be completed. Blue are the tasks that are yet to start.*

| Task | Y1Q1 | Y1Q2 | Y1Q3 | Y1Q4 | Y2Q1 | Y2Q2 | Y2Q3 | Y2Q4 | Status |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | Completed |
| 2 | | | | | | | | | Completed |
| 3 | | | | | | | | | Completed |
| 4 | | | | | | | | | Completed |
| 5 | | | | | | | | | On Schedule |

## Original Statement of Work Tasks

***FY12 Task 1: Isolation of stem cell populations from amniotic tissues. Months 1-4.***

a.) Isolate and characterize placental cells with surface markers characteristic of mesenchymal stem cells (MSCs) and hematopoietic cells. b.) Utilize expansion and differentiation tissue culture protocols to produce chondrocytes and compare the efficiency among cell types. c.) Determine appropriate concentrations of chondrogenic growth factors.

***FY12 Task 2: Characterization of cell surface markers on cell populations. Months 4-9.***

Characterize cultured cells using Flow Cytometry, and also determine which media is best for cell growth, and which conditioning media is best for cell differentiation to chondrocytes.

***FY12 Task 3: Expansion and chondrogenic differentiation. Months 9-18.***

Determine the ability of the MSCs to differentiate into a chondrocyte-like cell when seeded as 3D microtissues.

***FY12 Task 4: Seeding and growth of cell populations in 3-D matrix. Months 9-18.***

Determine if the alignment of chondrocytes could be controlled by mold design. Confirm that the new micro-mold designs can control cell-mediated tension in the intended direction. Confirm the direction of the tension environment.

***FY12 Task 5: Application of mechanical loading protocols. Months 12-24.***

Determine optimum dosimetry for uni- and biaxial strain in terms of tissue function.

**BODY**

**Hypothesis I**

Stem Cell Isolation, Expansion, and Differentiation

a. Isolate and characterize placental cells with surface markers characteristic of mesenchymal stem cells (MSCs) and hematopoietic cells.
b. Utilize expansion and differentiation tissue culture protocols to produce chondrocytes and compare the efficiency among cell types.
c. Determine appropriate concentrations of chondrogenic growth factors

Although our grant application focused on three-dimensional cultures and mechanotransduction, it is also important to compare the responses of MSCs derived from the placenta in traditional pellet cultures. Cultures from several different Passage #3 placental MSCs were tested on pellet culture model. Five million cells each were inoculated into Falcon cell culture tubes, allowed to aggregate for three days, and then subjected to 30 cycles per second rotational cultures over 21 days. Pellets were sampled at day 0, 3, 7, 14 and 21. A portion of the pellets were fixed and sectioned for histological analysis, Alcian blue and collagen immunohistochemical staining (**Figure 1**). A portion of the pellets were used for RNA isolation and evaluation of chondrocyte markers including aggrecan, Runx2, Sox 9, collagen 2 and collagen 10. The glycosaminoglycan concentration and DNA concentrations were also examined.

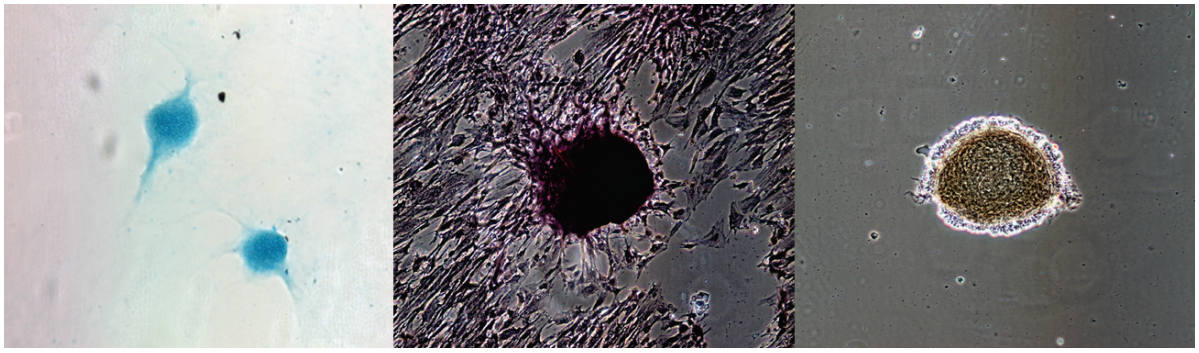

**Figure 1**: Amniotic MSCs in chondrogenic conditions (2D) formed tight nodes after 14 days. From left to right, nodes stained positive for alcian blue, safranin-O, and collagen II.

**KEY RESEARCH ACCOMPLISHMENTS**

- Successful isolation and culture of MSC's from amniotic and chorionic membranes of human placenta.
- FACS analysis characterization of MSC phenotype.
- We were able to expand the cell population to large numbers while maintaining MSC identity, making tissue engineering applications feasible.

**REPORTABLE OUTCOMES**

Abstracts accepted:

Ren, Y., J. Stabila, I Kurihara, J. Morgan, R.K. Aaron, J. Padbury. Isolation and surface characterization of mesenchymal stem cells (MSCs) derived from term human amnion. Exp Biol Med LB66, 9960, 2012.

Presentations:

"Isolation and Surface Characterization of MSCs Derived from Term Human Amnion", Experimental Biology Conference, San Diego, CA. 2012.

No manuscripts have been written, and no patents or licenses have been applied for. Tissues used in this project and the cells generated for it, have not been stored in any repositories. No funding has been applied for, based on this work.

## CONCLUSION

The cells are adherent to plastic, express common MSC surface markers (CD44, CD73, CD90, CD105), and are negative for common hematopoietic stem cell (CD34, CD117), macrophage (CD163, CD11b), and granulocyte (CD45) markers (Figure 1). In two dimensional cultures, chondrogenic potential (alcian blue, safranin-O, collagen II staining) and colony forming ability were established (**Figure 2**). We were able to expand the cell population to large numbers while maintaining MSC identity, making tissue engineering applications feasible.

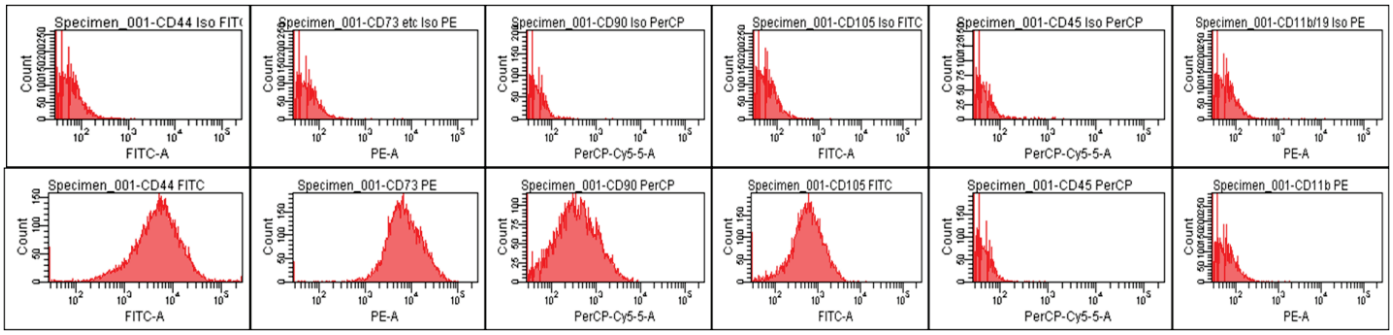


**Figure 2**: Selected FACS results from passage 3 amniotic MSCs. Positive for CD44, CD73, CD90, CD105 and negative for CD45 and CD11b.

## SO WHAT?

The results show that placental MSCs are a very suitable model for studying chondrogenesis using this traditional approach. There was early expression of the chondrocyte markers although they didn't seem to vary in a substantial way between days 3 and 21. Alcian blue staining indicating extracellular matrix appropriate to chondrocytes and collagen gene expression was seen and sustained. Perhaps most compelling was the gag/DNA ratio was between 8 and 15 by day 21 of culture. These results demonstrate using a traditional technique that placental MSCs are a suitable model for this project.

**Hypothesis II**

Precursor Cell Populations in 3-Dimensional Matrices

a. Create 3-D matrices of agarose containing relevant concentrations of chondrogenic growth factors.
b. Seed matrices with stem cells and differentiated chondrocytes and assess chondrogenesis.
c. Characterize the time course of replacement of agarose matrix.
d. Define principles that govern cell self-assembly and sorting thereby improving our ability to engineer tissues with appropriate spatial and functional properties.

To determine the ability of the MSCs to differentiate into a chondrocyte-like cell when seeded as 3D microtissues, MSCs were maintained in a low glucose Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAXTM –l (Invitrogen) and supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomyocin (p/s) at 5% $CO_2$. Monodispersed MSCs were then seeded into 2% agarose micromolds with differentiation media containing high glucose DMEM, 10% FBS, 1% p/s, 1 mM sodium pyruvate, 50 mM L-ascorbic acid 2-phosphate, 35 mM L-proline, 5 mg/ml ITS Premix, 10 ng/ml TGF-ß1, and 1.0 mM dexamethasone. After 1 week in the differentiation media, the microtissues were fixed and stained with a 1% alcian blue solution to determine the presence of strongly sulfated mucosubstances. All tissues stained bright blue indicating the presence of strongly sulfated mucosubstances(**Figure 1**).

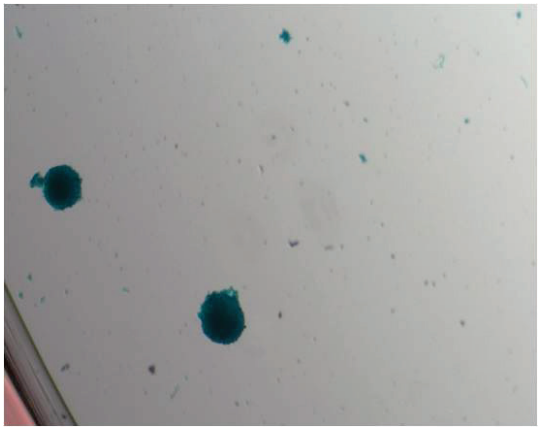

**Figure 1**: MSCs isolated from amniotic membrane and stained positive with 1% alcian blue within 1 week after seeding in 3D micromolds. Staining indicates the presence of highly sulfated mucosubstances.

Interestingly, in differentiation media, spheroids rapidly decreased in size with a 37% change in area between 1 and 2 weeks in culture (**Figure 2**). A LIVE/DEAD stain was used to determine the viability of these microtissues. The majority of cells within the spheroid were viable but there were many dead cells which had shed from the spheroid.

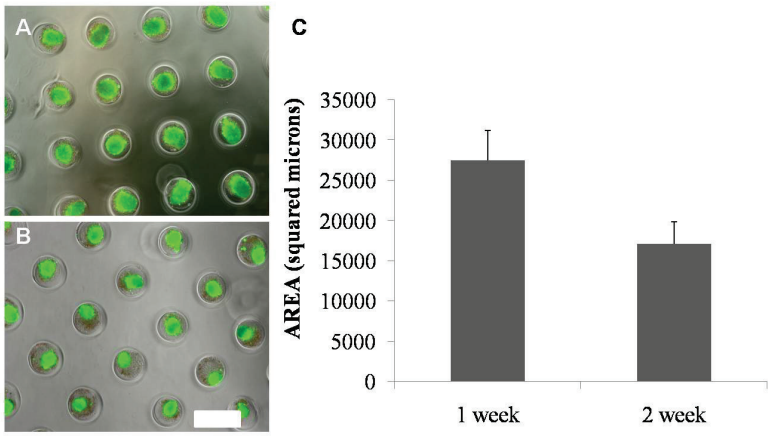


**Figure 2**: Although the majority of differentiated spheroids remained viable (green) at 1 (A) and 2 (B) weeks, there was a significant decrease in the area of the spheroids (37%) after 2 weeks when seeded in differentiation media with dead cells (red) shed from the spheroid.

Many differentiation media formulations have been used to differentiate MSCs into a chondrocyte like phenotype.[1] Other investigators have found that serum can cause cell apoptosis and they have used higher concentrations of ascorbic acid (200 mM compared to 50 mM) and lower concentrations of L-proline (0.35 mM compared to 35 mM).[2] To determine if these differences in media formulation could help maintain spheroid size, monodispersed MSCs were seeded into spheroid molds with a new differentiation media formulation which contained high glucose DMEM, 1% p/s, 1 mM sodium pyruvate, 200 mM L-ascorbic acid 2-phosphate, 0.35 mM L-proline, 5 mg/ml ITS Premix, 10 ng/ml TGF-ß1, and 1.0 mM dexamethasone (-10% FBS, higher L-ascorbic acid 2-phosphate, lower L-proline). In the new media formulation, spheroids maintained their size with spheroid area only changing by 11% between 1 and 2 weeks in culture (**Figure 3**). As well, compared to the old media formulation there were very few shed cells (**Figure 4**) and it took 4 weeks for spheroid area to decrease by 30% in new differentiation media compared to 1 week in the old formulation.

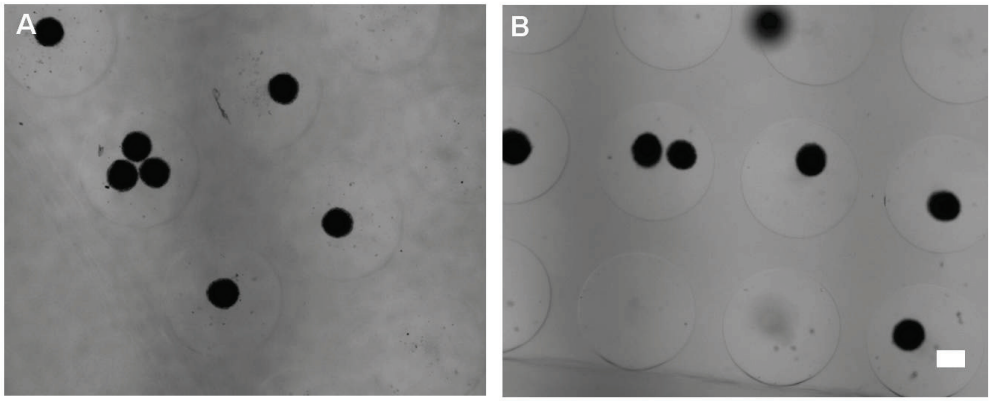


**Figure 3**: MSCs differentiated in new differentiation media maintained size. From 1 week (A) to 2 weeks (B) in culture MSC spheroids seeded in new differentiation media only changed area by 11% compared to 37% in old formulation.

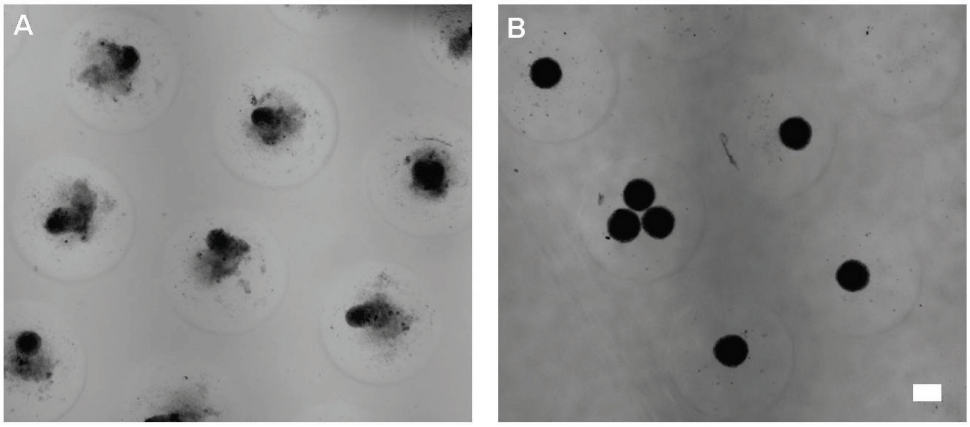


**Figure 4**: MSCs differentiated in new differentiation media formulation had better spheroid shape maintenance. MSCs seed in old media formulation (A) had smaller spheroids with many shed cells 2 weeks in culture compared to cells seeded with the new media formulation (B). Scale bar= 200 µm.

Native cartilage has 3 zones, the superficial, mid, and deep zone. We hypothesized that the alignment of the collagen fibers within the tissue contributed to its mechanical performance. In the superficial zone, collagen fibers are aligned in parallel to one another and the surface of the tissue, in the mid zone collagen fibers are unaligned and form a "basket weave," and in the deep zone the collagen fibers are aligned parallel to one another and perpendicular to the surface. These three zones have two major collagen fiber formations, straight fibers in parallel to one another (as seen in the superficial and deep zone) and fibers in a basket weave formation as in the mid zone. In micro-molded non adhesive hydrogels, the shape of the recess feature not only directs the self-assembly of cells into complex shapes, it also creates constraints to the tissue which cause stresses which lead to cell alignment and possibly the alignment of synthesized extracellular matrix (ECM). Fibroblast seeded into loop ended dogbones have been shown to generate a lot of tension which leads to cell alignment along one axis indicating that loop ended dogbones are under uni-axial strain.[3]

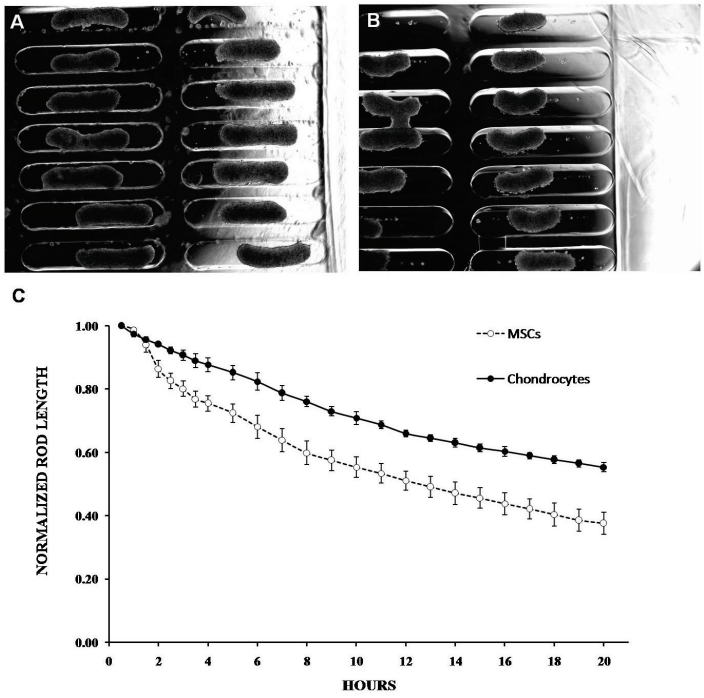


**Figure 5**: Both chondrocyte (A) and MSC (B) rod microtissues contract towards a spheroid, but at different rates and to different extents (C). After 20 hours in culture, chondrocytes contracted to 60% of their original length, while MSCs contracted to 40% of their original rod length.

A cell type's ability to form complex shaped microtissues is correlated to how well the cell type can maintain elongated rod shaped tissues. To determine the possibility of both forming complex shaped microtissues and using these microtissues as building blocks of larger chondrocyte structures we ran a rod contraction assay for both MSCs and human chondrocytes. Both chondrocyte and MSC rod microtissues contracted towards a spheroid, but at different rates and to different extents (**Figure 5**). After 20 hours in culture, chondrocyte rod microtissues contracted to 60% of their original length while MSC rods contracted to 40% of their original length. As a point of reference, fibroblast microtissues contract to 20% of their original length within this time frame and can still form and maintain (with some difficulty) stable complex shaped microtissues. This data also indicates that since chondrocyte microtissues contract slower and to a lesser extent than MSC microtissues, that to form complex shaped microtissues it may be beneficial to differentiate MSCs in 2D into a chondrocyte like phenotype prior to seeding into complex shaped geometries.

To determine if the alignment of chondrocytes could be controlled by mold design we developed different micro-molds to create tissues with uniaxial tension (loop-ended dogbones tissues), circumferential tension (toroid tissues), biaxial tension (cross tissues), equibiaxial tension (trampoline tissue), and multiaxial tension (triangle tissue) (**Figure 6**).

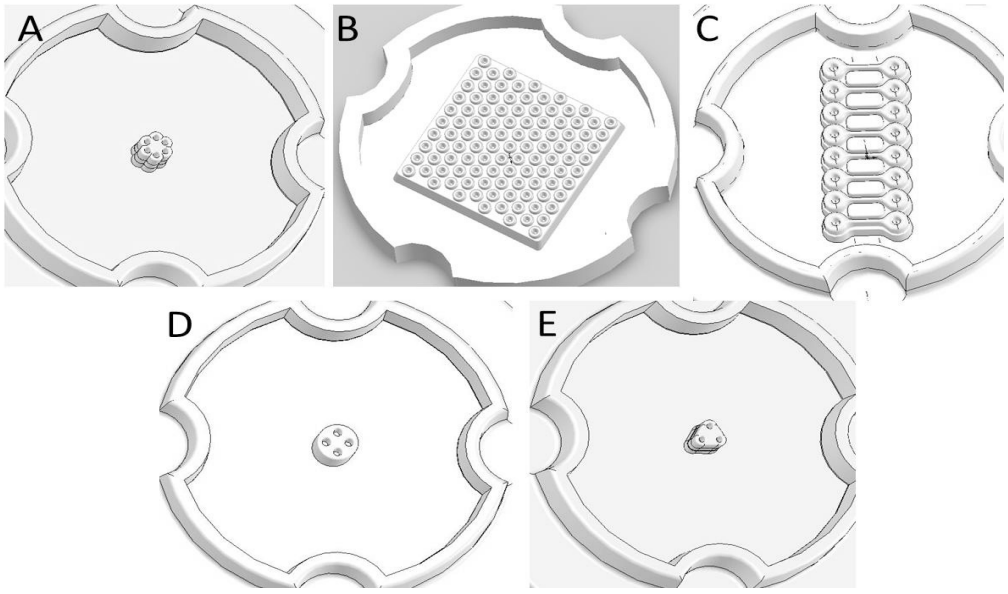


**Figure 6**: Micro-molds were designed to harness cell-derived forces to create equibiaxial (trampoline; A), circumferential (toroid; B), uniaxial (dogbone; C), biaxial (cross; D), and multiaxial tension (triangle; E) within the self-assembled tissue.

To confirm that the new micro-mold designs can control cell-mediated tension in the intended direction, we seeded chondrocytes into the micro-molds. Since the micro-molds are non-adhesive to cells, the cells rapidly self-assembled, and started to generate tension (**Figure 7A-E**). The microtissues were matured for four days, fixed, labeled with DAPI (nuclei stain) and fluorescently labeled phalloidin (F-actin stain), and viewed with confocal microscopy (**Figure 7F-J**).

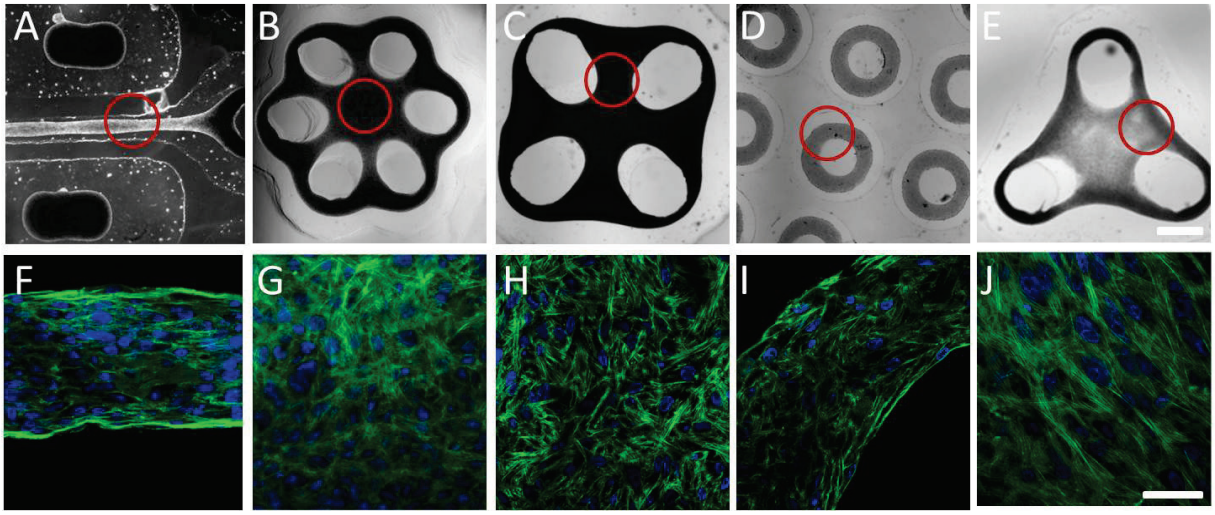


**Figure 7**: Chondrocytes were seeded into dogbone (A), trampoline (B), cross (C), toroid (D), and triangle (E) molds. The cells rapidly self-assembled, exerted tension which resulted in cell alignment. After 4 weeks, microtissues were fixed, their nuclei were labeled with DAPI (blue) and the f-actin was labeled with a fluorescent phalloidin (green), and the samples were imaged with confocal microscopy. Dogbones exhibited uniaxial nuclear alignment (F), trampolines had equibiaxial alignment (G), crosses had regions of uniaxial vertical (C) and uniaxial horizontal alignment, toroid had circumferential alignment (I), and triangles had alignment in the direction of the triangle "legs." (Scale bar= 300μm in panel E, and 100 μm in panel J)

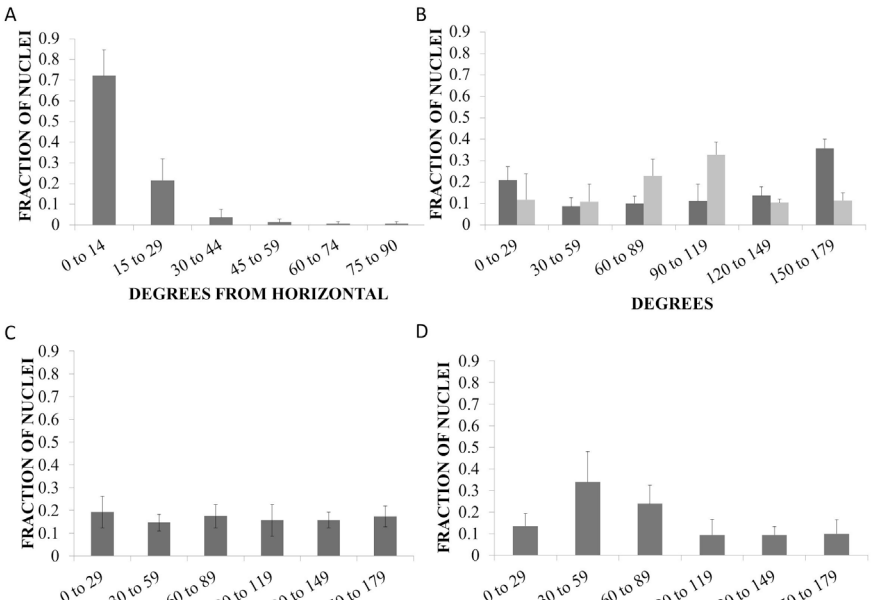


**Figure 8**: Micro-mold design controls nuclear alignment for different shaped microtissues. The angle of the major axis of the nuclei of dogbones (A), crosses (B), trampolines (C), and triangles (D) were quantified and binned in 15° bins for the dogbone, and 30° bins for the other shapes. Each shape had statistically significant more nuclei aligned in the bins predicted for each shape (0° to 14° from the horizontal for the dogbones; 0°, 90°, and 180° for the crosses; and 30-60° and 60-90° for the triangle). The fraction of nuclei in each of the bins for the trampoline was not statistically different from each other indicating that there was equibiaxial alignment.

To confirm the direction of the tension environment, we quantified the angle of the major axis of the nuclei of the cells in the dogbone, cross, trampoline, and triangle shaped microtissues (**Figure 8**). For the dogbone, it is expected that the cells would align uniaxially, and thus be parallel with the horizontal. Nuclei analysis showed that 72 + 13% of the cells were aligned between 0° to 14° from the horizontal with an average nuclei angle of 10 + 3°. For the cross, it is expected that there would be regions in which the cells are aligned around 0°and 180° and also regions in which the cells would align at 90°. Nuclei analysis indicated that there were statistically more cells aligned in these directions than the others ($p<0.05$) with peaks in the nuclei distribution at 0-30°, 90-120°, and 150-180°. If the trampoline microtissue has equibiaxial tension, then the cells would align equally along all directions.

The trampoline tissue did have nuclei aligned in all directions (0-180°), with no statistically significant difference in the number of cells within each angle bin. For the triangle microtissue, peg constraints were placed at the vertices of an equilateral triangle. This should result in cells aligning at 60°. Nuclei analysis of the triangle showed that the number of nuclei distributed in the 30-60° angle bin and the 60-90° angle bin were statistically more than the other angle bins ($p<0.05$), but not different from each other indicating that there was cell alignment with the average nucleus major axis aligned at 72 + 12°. All structures remained stable through 4 weeks with no signs of tissue weakening or necking (Figure 9).

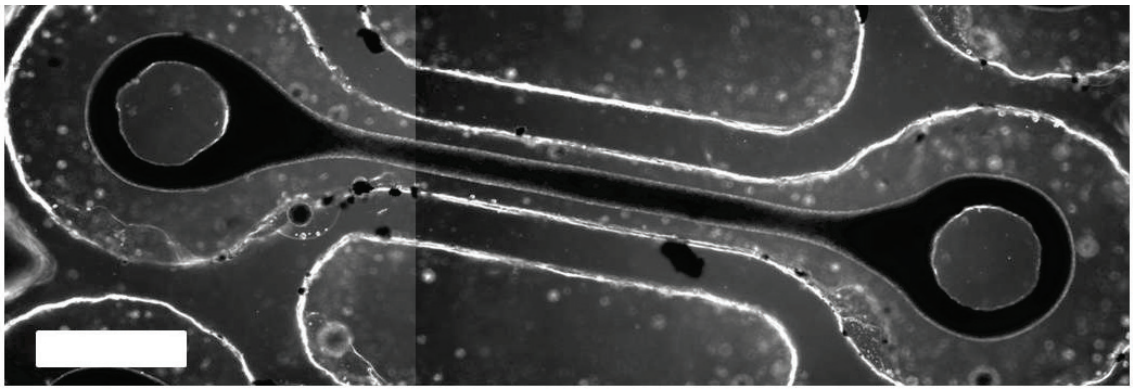

**Figure 9**: Chondrocytes seeded in differentiation media maintained a stable dogbone structure through 4 weeks in culture. Scale bar= 1 mm.

Chondrocytes synthesize and secrete collagen II. To determine if our micro-mold had control over the alignment of the ECM proteins synthesized by the cells, in addition to cell alignment, we stained for collagen. Chondrocytes were seeded into the toroid, dogbone, cross, trampoline, and triangle molds and the resulting microtissues were matured at 37°C and 5% $CO_2$ and fed chondrogenic media every 2-3 days. After 4 weeks, microtissues were fixed in 4% paraformaldehyde, and immunostainined for collagen II. In all of the tension environments, the chondrocytes produced a substantial amount of collagen II, which is a hallmark of cartilage (**Figure 10**). As well, the collagen II signal did intensify between 4 and 8 weeks in the dogbone samples indicating that the chondrocytes were still actively forming collagen II even at later culture time points (**Figure 11).**

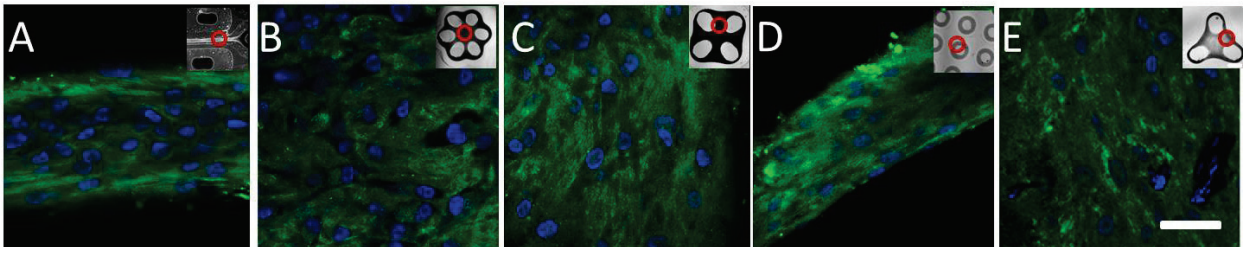


**Figure 10**: Collagen II alignment for the dogbone (uniaxial tension) (A), trampoline (equibiaxial tension )(B), cross (biaxial tension) (C), toroid (circumferential tension) (D), and triangle (multiaxial tension) (E) followed the direction of nuclear alignment. (Scale bar=100 μm).

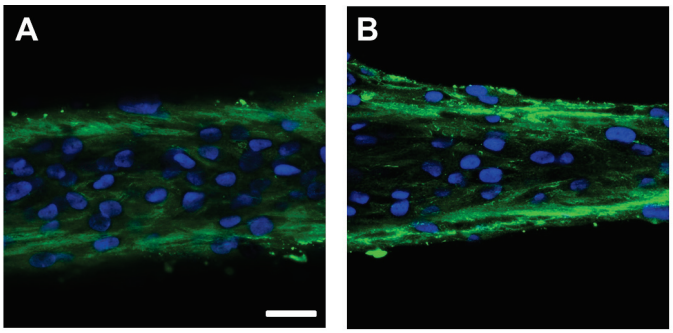


**Figure 11**: Collagen II expression increased in intensity between 4 (A) and 8 (B) weeks. Chondrocytes cultured in dogbone recesses with differentiation media were fixed and the nuclei labeled with DAPI and stained for collagen II with a rabbit anti-collagen II primary antibody and an anti-rabbit IgG FITC secondary (green). Collagen II signal (green) increased between 4 and 8 weeks. Scale bar=50 μm.

Unhealthy cartilage, or fibrocartliage, is marked by having a very high proportion of collagen I. Chondrocytes grown up for multiple passages on tissue culture are known to start synthesizing collagen I. To determine if our chondrocyte dogbones had a high proportion of collagen I, microtissues were fixed at 4 and 8 weeks and labeled with an antibody to collagen I. Confocal images revealed that although the dogbones did have some collagen I expression within 4 weeks, the signal decreased at 8 weeks, and the signal for collagen I was much smaller than that of collagen II (**Figure 12**). Specifically, collagen I staining was stronger at the outer surface of the tissue with very little staining towards the center of the tissue, and the total integrated fluorescent signal normalized to camera exposure was on average about 3x smaller than the normalized collagen II signal. Trampoline, toroid, cross, and triangle structures were also labeled for collagen I. Interestingly, the collagen I signal in these structures were similar to negative controls, implying that the chondrocytes synthesized very little collagen I in these shapes.

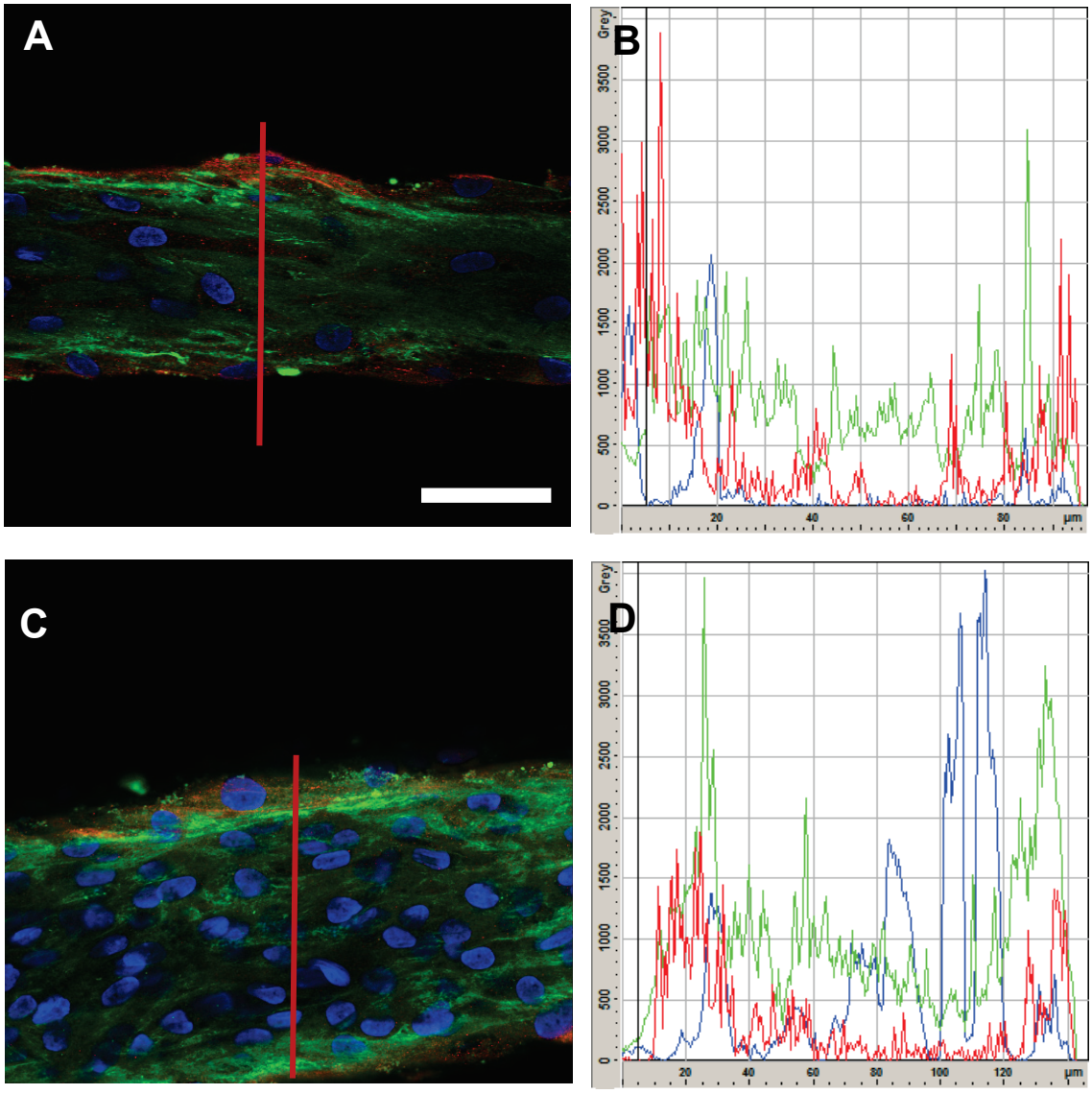


**Figure 12**: Chondrocytes cultured in dogbone recesses with differentiation media were fixed after 8 weeks and the nuclei was labeled with DAPI (blue) and stained for collagen II (green) with a rabbit anti-collagen II primary antibody and an anti-rabbit IgG FITC secondary and with a mouse anti-collagen I primary antibody and an anti-mouse IgG TRITC secondary (red) . Confocal imaging demonstrated that collagen II fibers were more abundant than collagen I at both the surface (A) and the middle of the tissue (C). Fluorescent intensity profiles through a single line in the tissue also demonstrate the relative intensity for the two collagens through the surface (B) and the middle of the tissues (D). Scale bar = 50 µm.

## KEY RESEARCH ACCOMPLISHMENTS

- Using just micro-mold design we were able to self-assemble chondrocytes into shapes that had many different cell as well as collagen alignments.
- Our microtissues were rich in collagen II, which is specific to cartilage and our shapes had a white glossy appearance similar to native cartilage (**Figure 13**). Not only were we able to recreate the uniaxial alignment and basket weave alignments of the three zones with our dogbone and trampoline structures, but we were also able to scale up our trampoline structures to more relevant sizes.
- The first trampoline we designed measured approximately 3.5 mm in diameter while the scaled-up version of this design measured approximately 11.5 mm in diameter.
- Chondrocytes did self-assemble these larger structures and remained stable through 8 weeks (**Figure 14**).

**Figure 13**: Chondrocyte dogbones had a white glossy appearance similar to native cartilage.

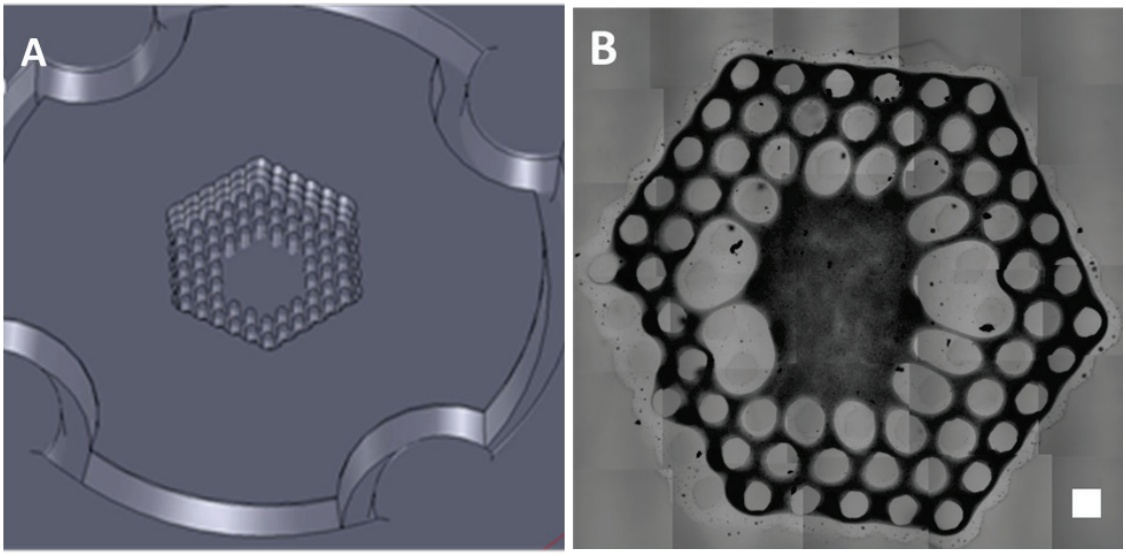


**Figure 14**: A scaled-up trampoline mold was designed (A) to create a tissue 11.5 mm in diameter. Chondrocytes formed a stable trampoline structures through 8 weeks. Scale bars= 0 .5 mm.

## REPORTABLE OUTCOMES

The data presented in this update is being prepared for a manuscript.

Funding from this grant supported the training of a postdoctoral fellow, Jacquelyn Schell, who has since received an Assistant Professor appointment at Brown University.

## CONCLUSIONS

We have demonstrated the ability to differentiate MSCs into a chondrocyte-like cell in 3D and to form chondrocytes into complex shaped tissues that could be used as building blocks for cartilage engineering. Once self-assembled into complex shaped microtissues, these cells align in the direction of tension which was dictated by the mold design and synthesize collagen II in the direction of tension. We have demonstrated the ability to recreate the alignment found in all three zones of cartilage and that these shapes can be scaled-up.

## SO WHAT?

Our goal was to use the 3D micromolded non-adhesive hydrogel technology to direct the formation of MSCs into complex shaped microtissues with geometries that may be suitable for the engineering of cartilage replacements. Towards this goal, we have determined that MSCs can self-assemble a number of different shaped microtissues. We found that these MSCs could be differentiated into chondrocyte nodules using the 3D micromold technology and that this technology gives a higher yield of differentiated nodules than tradition 2D differentiation methods.

**Hypothesis III**

Mechanical Strain of Cell-Matrix Constructs

a. Determine uniaxial dosimetry for compression.
b. Determine uniaxial dosimetry for shear.
c. Determine biaxial dosimetry.

Cells were harvested, washed, resuspended in media, and placed in conical vials for pellet culture. Each pellet had 500,000 cells and was formed by centrifuging for 10 minutes at 1500 RPM. The samples were cultured in a basal medium consisting of DMEM, high glucose with 1 mM sodium pyruvate, 1% ITS+ Premix, 0.4 mM L-proline, 0.1mM nonessential amino acids (NEAA), 50 µg/ml ascorbic acid (added fresh on day of use), 100 nM dexamethasone (added fresh on day of use), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2.5 µg/ml amphotericin B. Each sample was kept in the conical vial for the first three days of culture to allow pellet formation. Samples were then moved to 24-well plates with two mL of medium and placed on an orbital shaker at 60 RPM. Total culture time was two weeks. Experimental groups were cultured with different combinations of fetal bovine serum, recombinant human transforming growth factor-β1, and recombinant human transforming growth factor-β3 (Table 1).

Table 1: Experimental Groups

| Group | TGF-β1 (10 ng/mL) | TGF-β3 (10 ng/mL) | Fetal Bovine Serum (1%) |
|---|---|---|---|
| 1 | | | |
| 2 | | | X |
| 3 | X | | |
| 4 | X | | X |
| 5 | | X | |
| 6 | | X | X |

Glycosaminoglycan (GAG) results confirm some degree of chondrogenesis (**Figure 15**). The GAG/DNA values correspond to a range of approximately 4-8 µg GAG/sample, which is the low end of the detectable range. The serum-free, no growth factor group was statistically different from all other groups. No difference was seen between 1% FBS, TGF-β1, or TGF-β3.

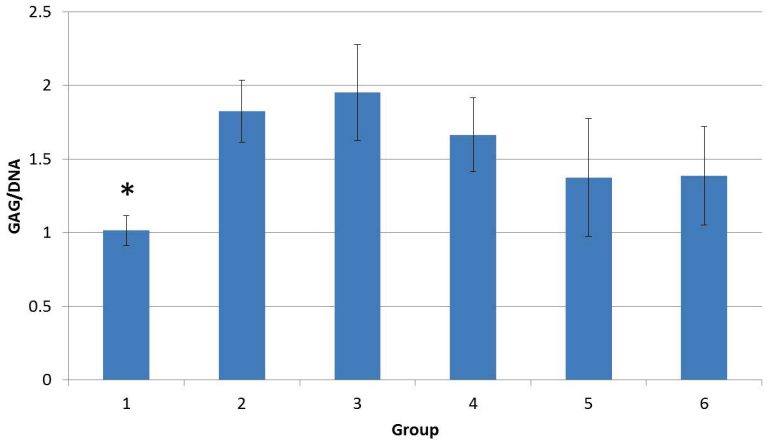


**Figure 15:** GAG/DNA (n=6) * $p<0.05$

Histology confirms GAG data. All groups appear to be negative for Safranin-O stain (**Figure 16**) and weakly positive for alcian blue stain (**Figure 17**). Upon examination at a higher magnification (**Figure 18**), some light matrix stain is visible, and some cells are stained blue. This indicates that early stages of differentiation may have occurred.

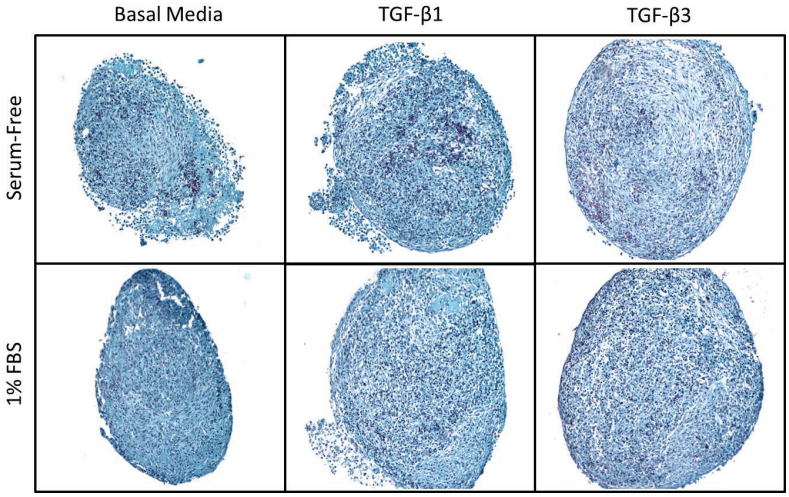


**Figure 16**: Safranin-O Staining, 10x

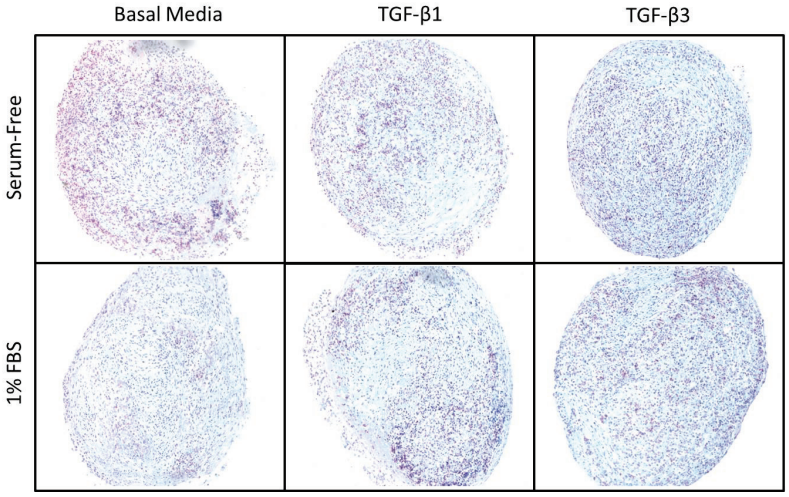


**Figure 17:** Alcian Blue Staining, 10x

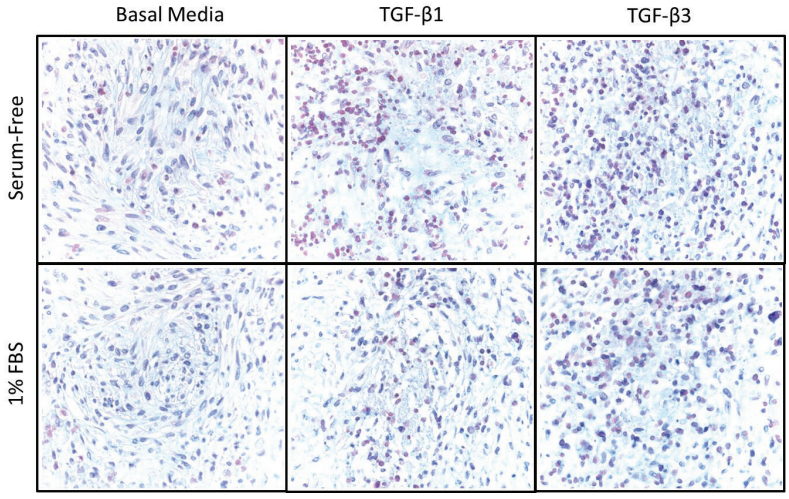


**Figure 18:** Alcian Blue Staining, 40x

Interestingly, all groups stained strongly positive for Collagen I (**Figure 19**). This indicates that these stem cells may have more of an osteogenic potential. Of course, it is possible that the media and culture conditions need to be further optimized for chondrogenesis. Although not as strong as Collagen I, all groups also stained positive for Collagen II (**Figure 20**).

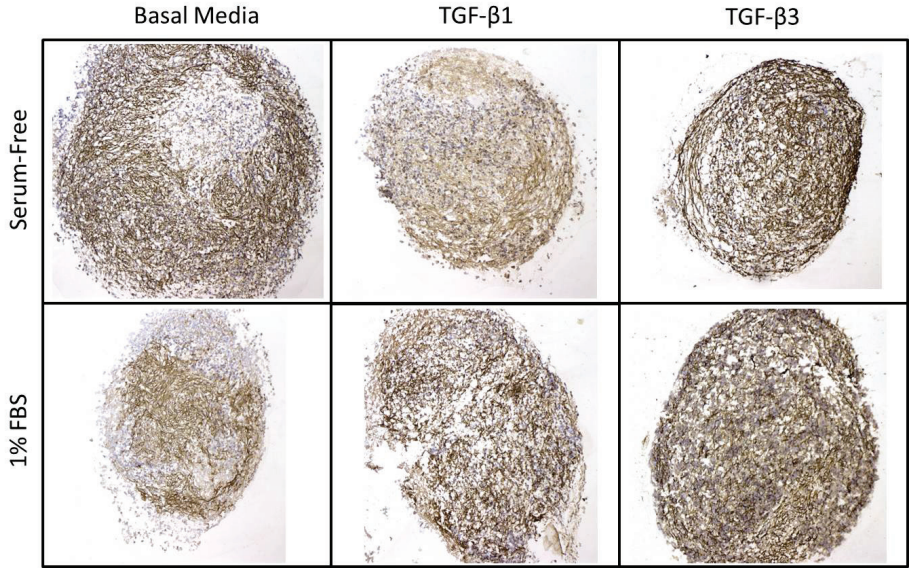


**Figure 19:** Collagen I Staining, 10x

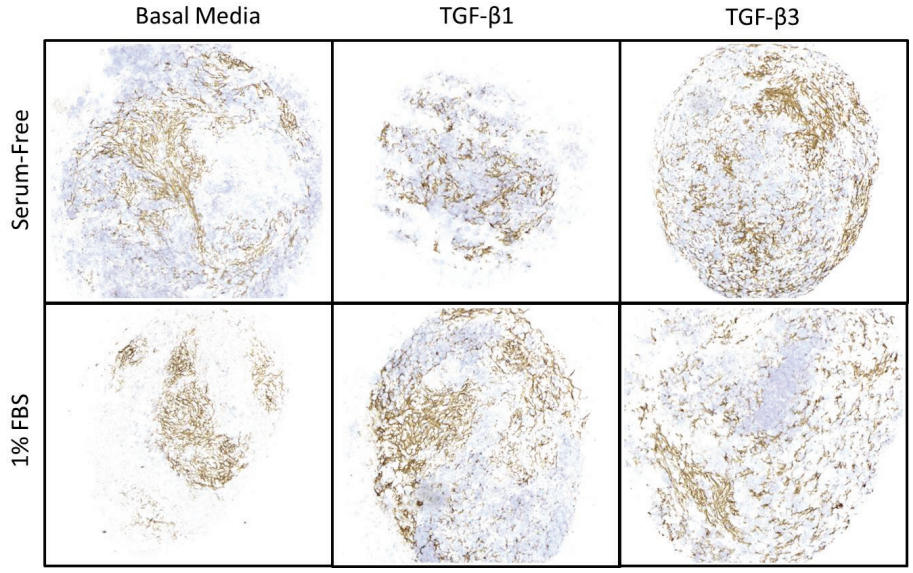


**Figure 20:** Collagen II Staining, 10x

We demonstrated the potential to create a cartilage biocomposite using these cells; however, cartilage-specific matrix accumulation was below clinically relevant levels. Dynamic loading combined with TGF-β1 supplementation increased glycosiminoglycan (GAG/DNA) and enhanced GAG distribution at day 42 in agarose hydrogels (**Figure 21**). DNA content was unaffected by loading within the basal media group (BM), however both chondrogenic media groups (CM) had significantly lower DNA content. TGF-β1 increased collagen/DNA relative to BM without growth factors while dynamic loading combined with TGF-β1 increased collagen/DNA compared to all groups. Histology showed pericellular staining for both types I and II collagen. We hypothesize that our results were a consequence of a mixed population of fibroblast-like cells and chondrocytes. It is possible that the fibroblast-like cells induced the type I collagen and relatively low GAG/DNA compared to other MSC sources.[4]

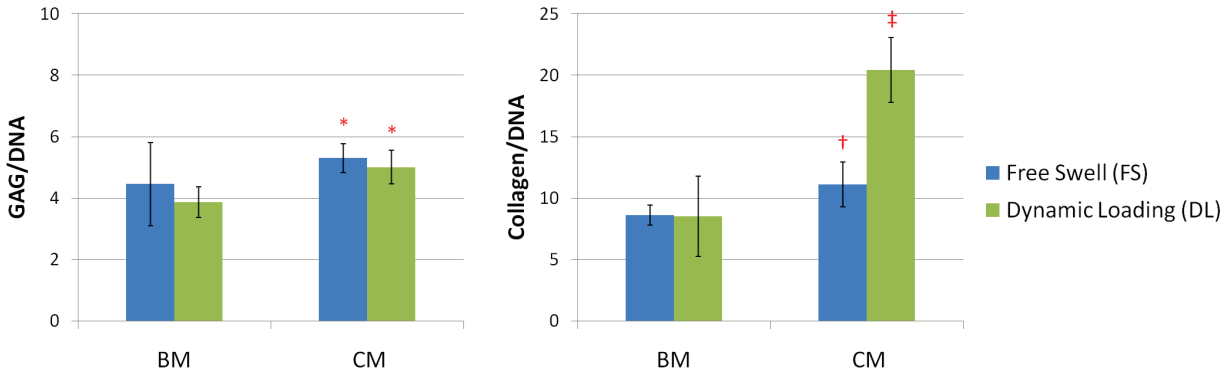


**Figure 21**: GAG/DNA (left) and collagen/DNA (right) comparing basal media (BM) and chondrogenic media (CM); *p<0.01 compared to BM-DL, †p<0.05 compared to BM-FS, ‡p<0.001 compared to all groups.

Our data suggest that the current surface marker expression profile does not reflect a homogeneous amniotic MSC population. In addition to MSCs, amnion and chorion contain populations of endothelial cells and fibroblasts. MSCs express many of the same surface markers as endothelial cells and fibroblasts, including CD29, CD44, CD73, CD90, CD105, and CD166.[5] Other tissue engineering studies utilizing placental MSCs have ignored this issue, potentially explaining the poor chondrogenesis.[6-8]

Dynamic mechanical loading has induced chondrogenesis, enhanced matrix deposition, and increased mechanical strength of MSC-laden hydrogels.[9-11] Our lab has shown that dynamic loading enhances GAG and collagen content in chondrocyte-agarose biocomposites at day 30 (**Figure 22**). To our knowledge, our initial dynamic loading experiment was the first to use amniotic MSCs.

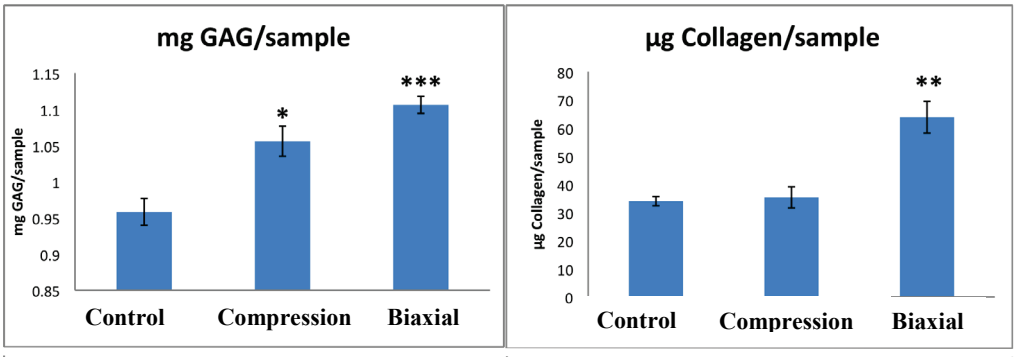


**Figure 22:** Biochemical analysis of dynamically loaded chondrocytes in agarose at day 30. *p<0.001 compared to control group, †p<0.001 compared to all groups.

Articular cartilage is normally relatively hypoxic *in vivo*. In order to enhance differentiation of amniotic MSC's into chondrocytes, *in vitro* oxygen gradients similar to those *in vivo* may be important. Previous results in our lab show that hypoxia more than triples the GAG content of synovial MSC loaded agarose at day 14 (**Figure 23**).

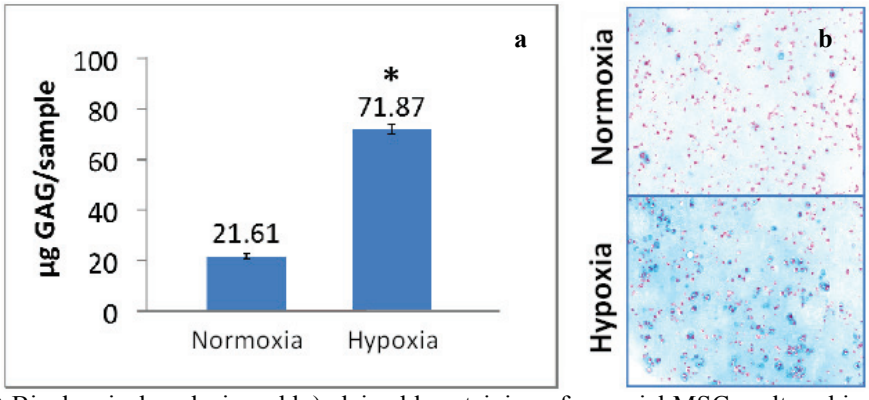


**Figure 23:** a.) Biochemical analysis and b.) alcian blue staining of synovial MSCs cultured in agarose for 14 days in hypoxic and normoxic conditions, *p<0.001.

A number of studies with mesenchymal stem cells from other sources in hypoxic conditions (1-5% $O_2$) have shown great promise. Hypoxia inducible factor (HIF)-1α is typically degraded in normoxic (21% $O_2$) conditions but has been found to remain stable and translocate to the nucleus at oxygen concentrations between 1 and 5%.[12] HIF-1α has been found to upregulate Sox9, a chondrocytic master transcription factor, as well as aggrecan and collagen II in bone marrow MSCs.[13, 14] Additionally, HIF-1α downregulates the fibrocartilage markers collagen I and X and decreases alkaline phosphatase activity.[15] Interestingly, bone marrow MSCs embedded in agarose hydrogels supplemented with TGF-ß3 and cultured in hypoxic conditions show higher GAG and collagen content than normoxic controls. Histological analysis of hypoxic constructs also demonstrate better distribution of GAG and collagen II than their normoxic counterparts.[16] Together, these studies suggest that culturing of amniotic MSC agarose constructs in hypoxia may result in a more chondrogenic matrix.

Our lab has recently developed a novel method to quantify the angle and anisotropy of the collagen fiber matrix in cartilage-like constructs. Sample cross-sections are imaged at 11kx using a transmission electron microscope (TEM). Images at varying depths are processed using a standardized ImageJ method and then analyzed using fast Fourier transform (FFT). The raw FFT output is a plot with angle of orientation on the x-axis and a unitless intensity value on the y-axis. An overlay of 3 plots along with representative TEM images is shown in **Figure 24**. The peak locations indicate the predominant fiber orientation in the image, and the peak height is a relative measure of anisotropy in that image. Angle and anisotropy values are extracted from these FFT plots, then averaged and plotted versus sample depth. In native porcine cartilage, we have used this method to differentiate among the 3 histological zones based on collagen fiber angle and anisotropy (**Figure 25**).

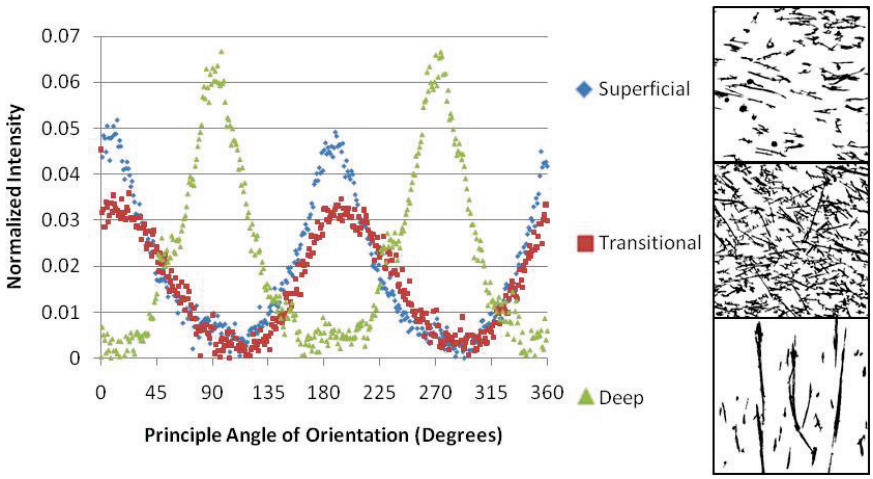


**Figure 24**: Normalized FFT data with corresponding binary fiber images.

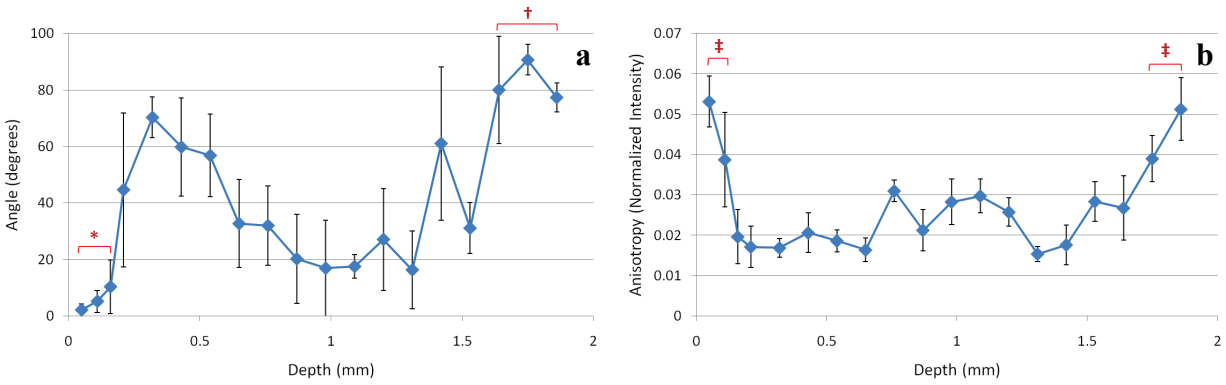


**Figure 25**: Relationship between collagen fiber angle (a) and anisotropy (b) to cartilage tissue depth in native immature porcine cartilage. N=5 images per data point on graph. Points grouped together into distinct zones, based on angle or anisotropy. *p<0.05 compared to all other points, †p<0.01 compared to all other points, ‡p<0.05 compared to all other points.

## KEY RESEARCH ACCOMPLISHMENTS

- Dynamic loading combined with TGF-β1 supplementation increased glycosiminoglycan (GAG/DNA) and enhanced GAG distribution at day 42 in agarose hydrogels.
- Dynamic loading enhances GAG and collagen content in chondrocyte-agarose biocomposites at day 30.
- Developed a novel method to quantify the angle and anisotropy of the collagen fiber matrix in cartilage-like constructs.

## REPORTABLE OUTCOMES

Abstract accepted:

Stefani R, Gonzalez D, Stabila J, Ren N, Padbury J, Aaron R."Human Amniotic Mesenchymal Stem Cells Suspended in Agarose Respond to TGF-β1 and Dynamic Compression: New Advances in Cartilage Tissue Engineering". Orthopaedic Research Society, San Antonio, TX. 2013

Abstract published:

Stefani, R., Aaron, R.K., Bilgen, B. Quantitative analysis of the depth-dependent ultrastructure of articular cartilage. Trans. Ortho. Res. Soc. 37: 1777; 2012.

Presentations:

"Surgical Solutions: Repair, Regeneration, and Replacement in Joint Diseases", Brown Alpert Medical School, Mini Med School Conference, Providence, RI. 2012.

"Osteoarthritis: Definitions and New Research". Osteoarthritis and You, Managing Pain, Living Well, Arthritis Foundation Seminar, Providence, RI. 2012.

"Quantification of the Depth-Dependent Ultrastructure of Articular Cartilage from Transmission Electron Micrographs: A Technique with Applications to Engineering Functional Tissue Replacements", Department of Orthopaedics Research Seminar, Brown University, Providence, RI. 2012.

## CONCLUSIONS

Cartilage matrix synthesis is below clinically relevant levels. By using the purified amniotic MSC population, our goal is to significantly increase cartilage matrix deposition (GAG and collagen II). We will investigate the effects of dynamic loading and hypoxia which have been proven to enhance chondrogenesis using other MSC sources.[17]

## SO WHAT?

The timing of the addition of growth factor application of mechanical loading is essential. Most functional tissue engineering studies, including our own, have had success with a two step approach. MSCs or chondrocytes seeded in a scaffold are supplemented with growth factor for 1 to 3 weeks of culture before they are subjected to loading for an additional 3 weeks.[17] This allows for chondrogenic differentiation and the formation of a chondroid matrix which is necessary for mechanotransduction.

We will examine the effects of dynamic loading on collagen fiber orientation. In tissue engineered constructs, dynamic loading has been shown to increase mechanical strength compared to free swelling controls.[18] However, a corresponding increase in GAG, collagen, and DNA content was not seen, suggesting that mechanical properties were enhanced by overall organization of matrix, not biochemical content. Additionally, long term *in vivo* loading of tissue engineered constructs induced reorganization of the collagen fiber network.[19] This study did not evaluate the functional properties of these samples, however.

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# APPENDIX

# ENGINEERING REPLACEMENT TISSUES WITH AMNIOTIC STEM CELLS
# STATEMENT OF WORK

## INTRODUCTION

The loss of bulk tissues in limb injuries, including segmental defects, is unfortunately common and results in extensive, complex wounds characterized by loss of several tissue types including, bone, cartilage, and muscle. Treatment of extensive multi-tissue defects poses many reconstructive challenges notably suitable cell sources, supportive 3-dimensional (3-D), spatially relevant matrices, and the engineering of tissues with functional properties appropriate to their biomechanical demands. The creation of such tissue replacements can be considered under the term, *functional tissue engineering*.

The long-term goals of this research program are to explore the inter-relationships of amniotic stem cell populations with 3-D matrices subjected to mechanical loading to fabricate a cartilage substitute with improved mechanical properties. We confirmed our original hypotheses that amniotic stem cells, which are widely available, can differentiate into multiple tissues and that the ability of cells to self-assemble and self-sort in 3-D matrices creates spatially robust tissue aggregates. We then investigated the chondrogenic potential of MSCs isolated from the amniotic and chorionic membranes seeded in agarose by assessing responses to growth factor delivery and compressive loading on matrix production. The following is our previous approved statement of work:

1. Stem Cell Isolation, Expansion, and Differentiation
   a. Isolate and characterize placental cells with surface markers characteristic of mesenchymal stem cells (MSCs) and hematopoietic cells.
   b. Utilize expansion and differentiation tissue culture protocols to produce chondrocytes and compare the efficiency among cell types.
   c. Determine appropriate concentrations of chondrogenic growth factors.
2. Precursor Cell Populations in 3-Dimensional Matrices
   a. Create 3-D matrices of agarose containing relevant concentrations of chondrogenic growth factors.
   b. Seed matrices with stem cells and differentiated chondrocytes and assess chondrogenesis.
   c. Characterize the time course of replacement of agarose matrix.
   d. Define principles that govern cell self-assembly and sorting thereby improving our ability to engineer tissues with appropriate spatial and functional properties.
3. Mechanical Strain of Cell-Matrix Constructs
   a. Determine optimum dosimetry for uni- and biaxial strain in terms of tissue function.

*These studies, while demonstrating the feasibility of our approach, raised new interesting questions that we feel need to be studied. Therefore, we are requesting to use the remaining funds in a no-cost extension to extend observations made in the first two years.*

## STATEMENT OF WORK FOR THE NO-COST EXTENSION

Each section begins with current observations which are justifications for continued work and concludes with work to be done (deliverables) in the no-cost extension.

### 1. STEM CELL ISOLATION, EXPANSION, AND DIFFERENTIATION

We have described a population of cells isolated from the amniotic and chorionic membranes that meet the minimal criteria for MSCs. The cells are adherent to plastic, express common MSC surface markers (CD44, CD73, CD90, CD105), and are negative for common hematopoietic stem cell (CD34, CD117), macrophage (CD163, CD11b), and granulocyte (CD45) markers (Figure 1). In two dimensional cultures, chondrogenic potential (alcian blue, safranin-O, collagen II staining) and colony forming ability were established. We were able to expand the cell population to large numbers while maintaining MSC identity, making tissue engineering applications feasible.

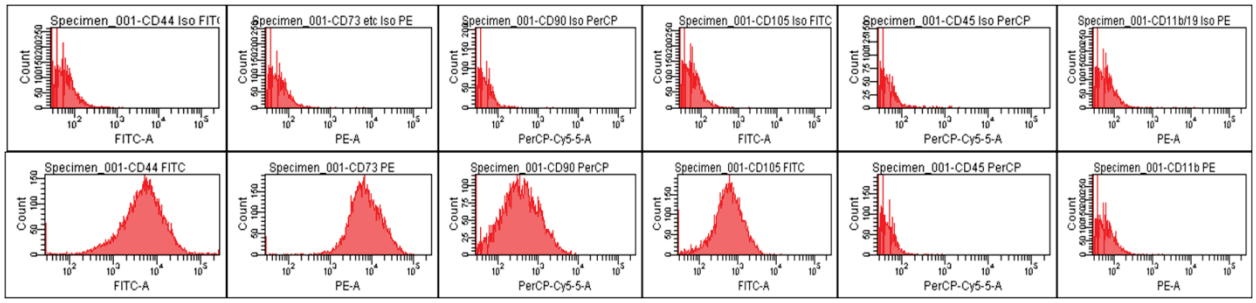


**Figure 1**: Selected fluorescence-activated cell sorting (FACs) results from passage 3 amniotic MSCs. Results were positive for CD44, CD73, CD90, CD105, and negative for CD45 and CD11b.

We demonstrated the potential to create a cartilage biocomposite using these cells; however, cartilage-specific matrix accumulation was below clinically relevant levels. Dynamic loading combined with TGF-β1 supplementation increased glycosiminoglycan (GAG/DNA) and enhanced GAG distribution at day 42 in agarose hydrogels (Figure 2). DNA content was unaffected by loading within the basal media group (BM), however both chondrogenic media groups (CM) had significantly lower DNA content. TGF-β1 increased collagen/DNA relative to BM without growth factors while dynamic loading combined with TGF-β1 increased collagen/DNA compared to all groups. Histology showed pericellular staining for both types I and II collagen. We hypothesize that our results were a consequence of a mixed population of fibroblast-like cells and chondrocytes. It is possible that the fibroblast-like cells induced the type I collagen and relatively low GAG/DNA compared to other MSC sources.[1]

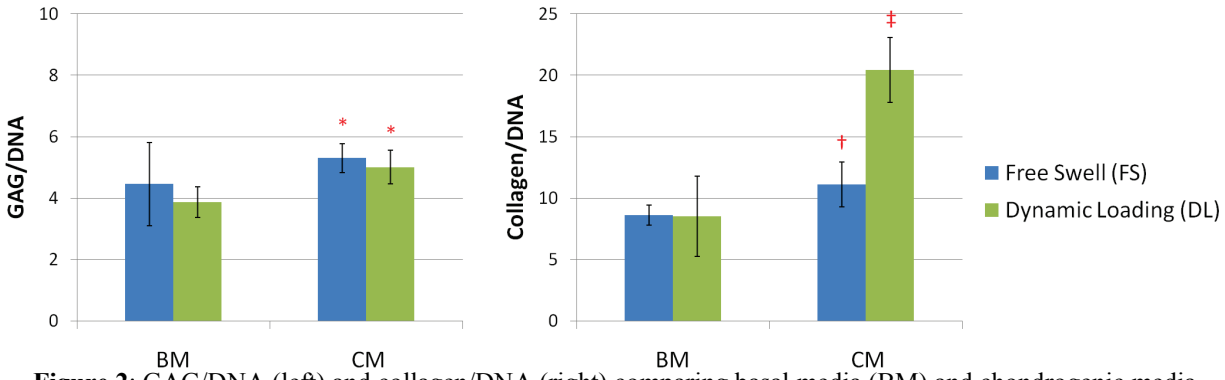


**Figure 2**: GAG/DNA (left) and collagen/DNA (right) comparing basal media (BM) and chondrogenic media (CM); *p<0.01 compared to BM-DL, †p<0.05 compared to BM-FS, ‡p<0.001 compared to all groups.

**Observations to be pursued**

Our data suggest that the current surface marker expression profile does not reflect a homogeneous amniotic MSC population. In addition to MSCs, amnion and chorion contain populations of endothelial cells and fibroblasts. MSCs express many of the same surface markers as endothelial cells and fibroblasts, including CD29, CD44, CD73, CD90, CD105, and CD166.[2] Other tissue engineering studies utilizing placental MSCs have ignored this issue, potentially explaining the poor chondrogenesis.[3-5]

**Work to be done in the extension period**

***Confirm the MSC Identity and Optimize Purity of Amniotic Derived Cells***

In the extension period, we will characterize amniotic MSCs using an expanded set of surface markers, and if necessary, magnetically sort the cells using one or more of these markers. CD146 is a promising surface marker for identifying MSC source; 92% of bone marrow MSCs and only 5% of fibroblasts have been shown to be CD146 positive at passage 2.[2] Additionally, CD146 positive umbilical cord MSCs had higher GAG content at day 21 pellet culture compared to bone marrow MSCs, suggesting a possible link to chondrogenesis.[6] Another marker of interest, CD49d, is expressed on MSCs and not on endothelial cells or hematopoietic stem cells.[7] We hypothesize that selecting CD146 and CD49d will significantly purify our stem cell population and enhance the chondrogenic potential of amniotic MSCs.

## 2. MECHANICAL STRAIN OF CELL-MATRIX CONSTRUCTS

Dynamic mechanical loading has induced chondrogenesis, enhanced matrix deposition, and increased mechanical strength of MSC-laden hydrogels.[8-10] We will apply dynamic biaxial loading to biocomposites using our mechanical loading device has shown in previous reports. Our lab has shown that dynamic loading enhances GAG and collagen content in chondrocyte-agarose biocomposites at day 30 (Figure 3). To our knowledge, our initial dynamic loading experiment (described in task 1) was the first to use amniotic MSCs.

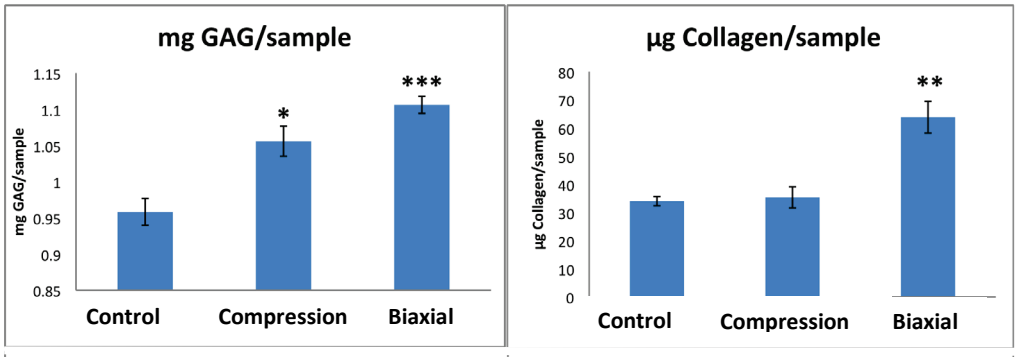


**Figure 3:** Biochemical analysis of dynamically loaded chondrocytes in agarose at day 30. *p<0.001 compared to control group, †p<0.001 compared to all groups.

Articular cartilage is normally relatively hypoxic *in vivo*. In order to enhance differentiation of amniotic MSC's into chondrocytes, *in vitro* oxygen gradients similar to those *in vivo* may be important. Previous results in our lab show that hypoxia more than triples the GAG content of synovial MSC loaded agarose at day 14 (Figure 4). A number of studies with mesenchymal stem cells from other sources in hypoxic conditions (1-5% $O_2$) have shown great promise. Hypoxia inducible factor (HIF)-1α is typically degraded in normoxic (21% $O_2$) conditions but has been found to remain stable and translocate to the nucleus at oxygen concentrations between 1 and 5%.[11] HIF-1α has been found to upregulate Sox9, a chondrocytic master transcription factor, as well as aggrecan and collagen II in bone marrow MSCs.[12, 13] Additionally, HIF-1α downregulates the fibrocartilage markers collagen I and X and decreases alkaline phosphatase activity.[14] Interestingly, bone marrow MSCs embedded in agarose hydrogels supplemented with TGF-ß3 and cultured in hypoxic conditions show higher GAG and collagen content than normoxic controls. Histological analysis of hypoxic constructs also demonstrate better distribution of GAG and collagen II than their normoxic counterparts.[15] Together, these studies suggest that culturing of amniotic MSC agarose constructs in hypoxia may result in a more chondrogenic matrix.

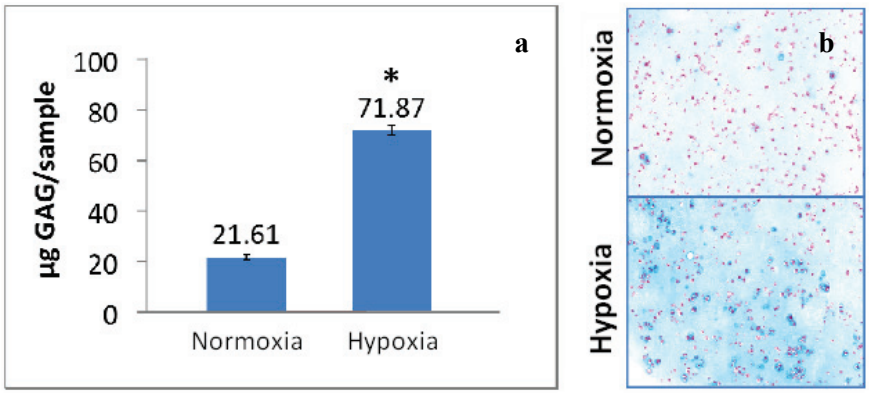


**Figure 4:** a.) Biochemical analysis and b.) alcian blue staining of synovial MSCs cultured in agarose for 14 days in hypoxic and normoxic conditions, *p<0.001.

## Observations to be pursued

Cartilage matrix synthesis is below clinically relevant levels. By using the purified amniotic MSC population, our goal is to significantly increase cartilage matrix deposition (GAG and collagen II). We will investigate the effects of dynamic loading and hypoxia which have been proven to enhance chondrogenesis using other MSC sources.[16]

## Work to be done in the extension period

### *Optimize Matrix Deposition in Amniotic MSC Loaded Biocomposites*

We will optimize culture conditions, and then we will establish that constructions respond favorably to dynamic mechanical loading. In both phases, amniotic MSCs will be seeded in agarose hydrogels. In the first phase, constructs will be cultured in defined, serum-free chondrogenic media, and low oxygen tension (2% $O_2$) or normal oxygen tension (21% $O_2$) for 3 weeks. We will then evaluate the stage of chondrogenic differentiation and degree of matrix production based on gene expression profile (aggrecan, collagen II, cartilage oligomeric matrix protein, Sox9), and quantitative assays for DNA and matrix proteins, (GAG, and types I and II collagen). A second 3 weeks of culture will then be performed: half of the samples will remain in free swelling conditions while the other half is conditioned by dynamic mechanical compression for 3 hours/day. Constructs will again be evaluated for chondrogenic gene expression, biochemical content, and histology.

The timing of the addition of growth factor application of mechanical loading is essential. Most functional tissue engineering studies, including our own, have had success with a two step approach. MSCs or chondrocytes seeded in a scaffold are supplemented with growth factor for 1 to 3 weeks of culture before they are subjected to loading for an additional 3 weeks.[16] This allows for chondrogenic differentiation and the formation of a chondroid matrix which is necessary for mechanotransduction.

## 3. Investigate the Relationship between Dynamic Loading, Collagen Fiber Architecture, and Mechanical Strength of Amniotic MSC Biocomposites

Our lab has recently developed a novel method to quantify the angle and anisotropy of the collagen fiber matrix in cartilage-like constructs. Sample cross-sections are imaged at 11kx using a transmission electron microscope (TEM). Images at varying depths are processed using a standardized ImageJ method and then analyzed using fast Fourier transform (FFT). The raw FFT output is a plot with angle of orientation on the x-axis and a unitless intensity value on the y-axis. An overlay of 3 plots along with representative TEM images is shown in Figure 5. The peak locations indicate the predominant fiber orientation in the image, and the peak height is a relative measure of anisotropy in that image. Angle and anisotropy values are extracted from these FFT plots, then averaged and plotted versus sample depth. In native porcine cartilage, we have used this method to differentiate among the 3 histological zones based on collagen fiber angle and anisotropy (Figure 6).

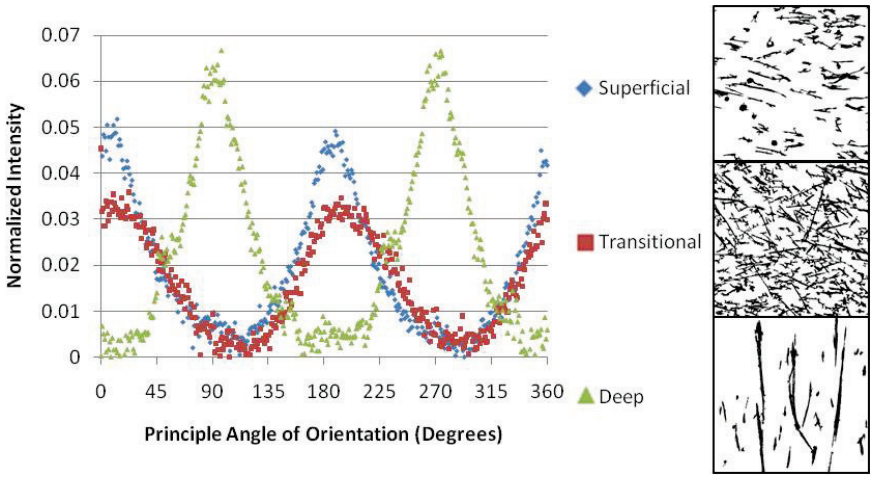


**Figure 5**: Normalized FFT data with corresponding binary fiber images.

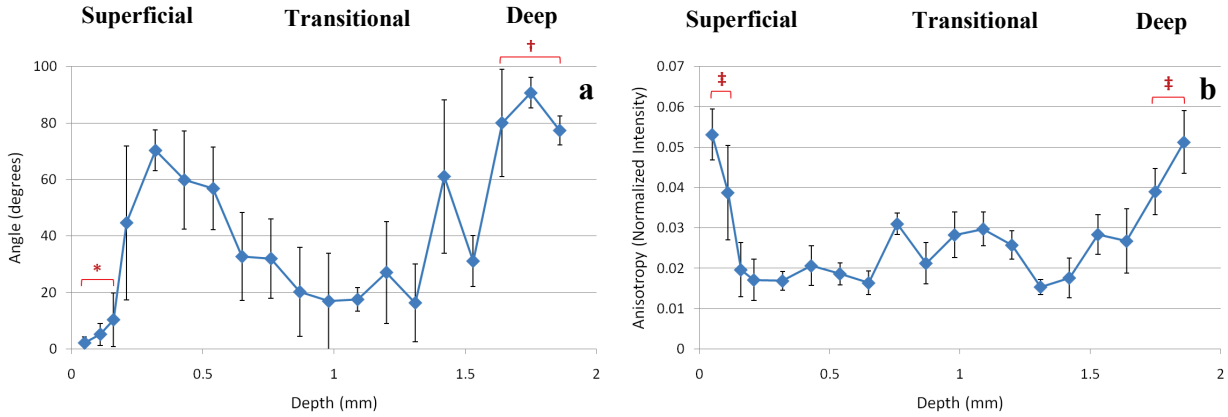


**Figure 6**: Relationship between collagen fiber angle (a) and anisotropy (b) to cartilage tissue depth in native immature porcine cartilage. N=5 images per data point on graph. Points grouped together into distinct zones, based on angle or anisotropy. *p<0.05 compared to all other points, †p<0.01 compared to all other points, ‡p<0.05 compared to all other points.

## Observations to be pursued

We will examine the effects of dynamic loading on collagen fiber orientation. In tissue engineered constructs, dynamic loading has been shown to increase mechanical strength compared to free swelling controls.[17] However, a corresponding increase in GAG, collagen, and DNA content was not seen, suggesting that mechanical properties were enhanced by overall organization of matrix, not biochemical content. Additionally, long term *in vivo* loading of tissue engineered constructs induced reorganization of the collagen fiber network.[18] This study did not evaluate the functional properties of these samples, however.

## Work to be done in the extension period

### *Mechanical Strength and Collagen Fiber Alignment*

Sample mechanical strength will be evaluated by measuring equilibrium compressive modulus and dynamic modulus. We hypothesize that *in vitro* biaxial loading enhances functional properties of biocomposites not only by increasing matrix deposition, but also by inducing changes in the zonal collagen architecture. Using the protocol established in task 2 as a starting point, we will apply dynamic compressive and shear strain to biocomposites using our mechanical loading device. Gene expression and biochemical content will be evaluated. Additionally, using the previously described FFT method, collagen organization will be quantified.

## TIMELINE

The chart below provides a timeline of the original tasks and their status as well as the new tasks starting with Year 3 Quarter 1 as stated in the new SOW. Green when task is completed. Light green for task that is on schedule and active. Yellow when it is delayed; include a red line showing when it is to start and when you anticipate it to be completed. Blue are the tasks that are yet to start.

| Task | Y1Q1 | Y1Q2 | Y1Q3 | Y1Q4 | Y2Q1 | Y2Q2 | Y2Q3 | Y2Q4 | Y3Q1 | Y3Q2 | Y3Q3 | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Task 1** | | | | | | | | | | | | **Completed** |
| **New Task 1** | | | | | | | | | | | | **Yet to start** |
| **Task 2** | | | | | | | | | | | | **Completed** |
| **Task 3** | | | | | | | | | | | | **On Schedule** |
| **New Task 2** | | | | | | | | | | | | **Yet to start** |
| **Task 4** | | | | | | | | | | | | **On Schedule** |
| **Task 5** | | | | | | | | | | | | **On Schedule** |
| **New Task 3** | | | | | | | | | | | | **Yet to start** |

## NEW TASKS

1. Stem Cell Isolation, Expansion, and Differentiation (months 1-3)
   *a) Confirm the MSC Identity and Optimize Purity of Amniotic Derived Cells*
2. Mechanical Strain of Cell-Matrix Constructs (months 1-4)
   *b) Optimize Matrix Deposition in Amniotic MSC Loaded Biocomposites*
3. Investigate the Relationship between Dynamic Loading, Collagen Fiber Architecture, and Mechanical Strength of Amniotic MSC Biocomposites (months 1-7)
   *c) Mechanical Strength and Collagen Fiber Alignment*

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